ORIGINAL ARTICLE

Carbapenem Resistance in Gram-negative Bacteria in South-western Nigeria: The Role of Extended-spectrum β-lactamase CTX-M-15

DO Ogbolu[1, 2], MA Webber[2]

ABSTRACT

Objective: *To determine the role of extended-spectrum β-lactamases in carbapenem-resistant Gram-negative bacteria from south-western Nigeria.*

Methods: *Twenty-seven carbapenem-resistant isolates that were found to be non-carbapenemase producers (15* Escherichia coli*, 9* Klebsiella pneumoniae *and 3* Pseudomonas aeruginosa*) were further studied. These isolates were subjected to analysis including phenotypic and genotypic detection of various β-lactamases, efflux activity, outer membrane protein, plasmids replicon typing, detection of transferable genes and resistances and typing using random amplified polymorphic DNA tests.*

Results: *No isolates demonstrated de-repression of efflux, but all showed either complete loss or reduced production of outer membrane proteins. Transconjugants from these strains contained various genes including plasmid-mediated quinolone resistance and extended-spectrum beta-lactamases. All the transconjugants carried the* bla*CTX-M-15 gene. The transconjugants had varying minimum inhibitory concentrations of carbapenems ranging from 0.03 µg/ml to 8 µg/ml. Varying resistances to other antimicrobial agents were also transferred with the plasmids. The donor isolates were not clonally related by molecular typing.*

Conclusion: *Resistance to carbapenem antibiotics in this sample was not mediated only by carbapenemases but also by production of extended-spectrum β-lactamases (largely CTX-M-15), accompanied by protein loss. This was an important mechanism underpinning carbapenem resistance in these clinical isolates of various species.*

Keywords: Carbapenem, extended-spectrum beta-lactamase, Gram-negative bacteria, Nigeria, resistance

Resistencia al carbapenem en las bacterias gramnegativas en Nigeria Suroccidental: papel de la betalactamasa de espectro extendido tipo CTX-M-15

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RESUMEN

Objetivo: *Determinar el papel de las betalactamasas de espectro extendido en la resistencia al carbapenem en las bacterias gramnegativas en Nigeria.*

Métodos: *Veintisiete aislados resistentes al carbapenem que fueron hallados productores de no carbapenemasas (15* Escherichia coli*, 9* Klebsiella pneumoniae*, y 3* Pseudomonas aeruginosa*)*

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fueron estudiados con mayor profundidad. Estos aislados fueron sometidos a análisis incluyendo la detección fenotípica y genotípica de varias betalactamasas, la actividad de eflujo, las porinas de la membrana externa, la tipificación del replicón plasmídico, la detección de genes transferibles y resistencias y tipificación usando pruebas de ADN polimórficas amplificadas aleatorias.

Resultados: *Ninguno de los aislamientos mostró desrepresión de eflujo, pero todos demostraron la pérdida completa o la producción reducida de porinas externas de la membrana. Los transconjugantes de estas cepas contenían varios genes incluyendo resistencia a la quinolona mediada por plásmidos y betalactamasas de espectro extendido. Todos los transconjugantes portaban el gen blaCTX-M-15. Los transconjugantes tenían diversas concentraciones inhibitorias mínimas de carbapenemas que oscilaban entre 0.03 μg/ml y 8 μg/ml. Varias resistencias a otros agentes antimicrobianos fueron también transferidas con los plásmidos. Los aislamientos del donante no estuvieron relacionados clonalmente por tipificación molecular.*

Conclusión: *La resistencia al antibiótico carbapenem en esta muestra no fue mediada solamente por las carbapenemasas, sino también por la producción de betalactamasas de espectro extendido (en gran parte CTX-M-15), acompañado por pérdida de porina. Éste era un mecanismo importante que sustentaba la resistencia al carbapenem en estos aislados clínicos de varias especies.*

Palabras clave: Carbapenem, betalactamasa de espectro extendido, bacterias gramnegativas, Nigeria, resistencia

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INTRODUCTION

Gram-negative bacteria (GNB) are increasingly resistant to antibiotics and particularly to broad-spectrum cephalosporins, because of the global spread of extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* and the emergence of *Enterobacteriaceae* and *P aeruginosa* producing high levels of AmpC cephalosporinase (1). Carbapenems are currently the only active β-lactams effective against these bacteria; this has led to an increase in their use not only for documented infections but also for empirical treatment of hospital-acquired infections. As a result, this has led to selective pressure for carbapenem resistance which has spread progressively (2), culminating with the worldwide rapid emergence of resistance to carbapenems during the 1990s in GNB (3). Of particular concern is the fact that carbapenem-resistant GNB are often resistant to other classes of antibiotics (such as aminoglycosides, fluoroquinolones and cephalasporins), leaving very few therapeutic options (4).

Carbapenem resistance in the *Enterobacteriaceae* has been ascribed mainly to enzymatic degradation by plasmid-borne carbapenemases of classes A, B and D (5). In addition, carbapenem resistance development in isolates producing CTX-M and other ESBLs (6) or plasmid-borne AmpC β-lactamases due to selection of mutants lacking expression of outer membrane porins has been observed (7). We had previously identified carbapenemases as a cause of carbapenem resistance in GNB from Nigeria (8). However, there still remained carbapenem-resistant isolates without production of a carbapenemase. Therefore, this study aimed to determine the role of extended-spectrum β-lactamases in carbapenem-resistant Gram-negative bacteria from south-western Nigeria.

MATERIALS AND METHODS

Bacterial isolates

The 27 GNB isolates used in this study were from our culture collection and had been found to be carbapenem-resistant. However, they did not produce a carbapenemase. These isolates were collected from a range of clinical sites from four tertiary hospitals in south-western Nigeria in 2012. Isolates comprised 15 *Escherichia coli* (*E coli*), 9 *Klebsiella pneumoniae* (*K pneumoniae*) and 3 *Pseudomonas aeruginosa* (*P aeruginosa*) and were identified using API 20E strips (bioMérieux, Basingstoke, United Kingdom (UK)) and conventional biochemcial tests.

Detection of β-lactamases

The production of ESBLs was identified by double disc synergy testing (9). AmpC β-lactamase production was also detected using cefepime and cefpodoxime

disks in combination with clavulanate as previously described (10).

Minimum inhibitory concentrations

The minimum inhibitory concentrations (MICs) of the donor strains including recipient strain to the three carbapenem drugs and other important antimicrobial agents were determined using the agar dilution method as described by Andrew (11). All runs included the control organisms: *E coli* (NCTC 10418) and *P aeruginosa* (NCTC 10662). A start and finish plate without antibiotics was also included as a growth control. Minimum inhibitory concentrations were determined on at least two separate occasions for each strain.

Random amplified polymorphic DNA and polymerase chain reaction typing

The epidemiological relationships among the 27 isolates belonging to the same major species of *E coli*, *K pneumoniae* and *P aeruginosa* were analysed by random amplified polymorphic DNA. The primers sequence and polymerase chain reaction (PCR) running conditions used were according to Vogel *et al* (12).

Transfer of resistance

In order to determine if ESBL resistance was transferable, conjugation experiments were carried out for a selection of 13 of the ESBL producers. The criteria were based on their susceptibility (MIC) profiles and clonality. Conjugation experiments were carried out by broth and filter mating assays with a donor to recipient ratio of 1:1, incubated for 12 hours at 37ºC using sodium azide-resistant *E coli* J-53 as a recipient cell. Suspensions of 200 µl of the 13 isolates were plated out onto selective plate containing sodium azide (100 µg/ml) and ampicillin (16 µg/ml). Controls included test isolates plated on sodium azide plates and recipient cells on separate plates containing sodium azide or ampicillin.

Measuring the activity of active efflux using Hoescht 33342 (bis-benzimide)

The efflux activity of bacteria (test isolates and control, MG1655) was determined by measuring the accumulation of the fluorescent dye Hoechst 33342 (bis-benzimide; 2.5 µM) in the presence or absence of the efflux pump inhibitor phenyl-arginine-beta-naphthylamide (PAßN, 40 mg/L). Measurements were taken at excitation and emission wavelengths of 350 nm and 460 nm, respectively, over 30 minutes using a FLUOstar OPTIMA (BMG Labtech, Aylesbury, UK), as previously described (13). The data were presented by plotting graphs to include standard deviation.

Analysis of outer membrane proteins

In order to investigate whether alteration in outer membrane permeability contributed to carbapenem resistance, we analysed the outer membrane proteins (OMPs) of seven isolates comprising *E coli* and *K pneumoniae* that were successfully transferred. Bacterial cells were grown in Mueller-Hinton broth to logarithmic phase and were lysed by sonication. Outer membrane proteins were extracted with sodium lauryl sarcosinate (Sigma, UK) and recovered by ultracentrifugation (14). Protein concentrations were determined with the Bradford protein assay kit (Sigma, UK) as described by the manufacturer. The OMP profiles were determined by SDS-PAGE using 12% SDS gels followed by Coomassie blue staining (Carlsbad, CA, United States of America). The presence and intensity of bands were visualized compared with wild type isolates of *E coli* (MG1655) and *K pneumoniae* (NCTC 9633), using a Syngene 'Geneious' Image analyser.

Detection of resistance genes

Polymerase chain reaction was used to detect transferable plasmids encoding resistance genes of entire sequences of known carbapenemase *bla* genes (NDM, KPC, OXA-48, VIM, IMP, SPM, SIM, GIM and GES (15–17)), ESBL *bla* genes (OXA, SHV, TEM, CTX-M and AmpC), as well as plasmid-mediated quinolone resistance (PMQR) genes, as previously described (18). Amplimers resulting from these PCR reactions were sequenced to confirm the identity and specific variant of each gene identified, and sequences were aligned to known reference sequences using ClustalW (www.ebi.ac.uk/Tools/clustalw2/index.html).

Replicon/incompatibility testing

Total DNA was generated using the Wizard Genomic DNA Purification System (Promega, Madison, WI, United States of America) for identification of plasmids according to 'Inc' type specific PCR (19).

RESULTS

The isolates were ESBL producers. The majority of the isolates were not clonally related by molecular typing. The *E coli* isolates were all from the same hospital while the *K pneumoniae* and *P aeruginosa* isolates were from two other hospitals.

Table 1: Minimum inhibitory concentrations and properties of donors and transconjugants

	Isolates																		
	S21		**U58**		**I88**		**I98**		**U61**		**S(U25)**		**S(U37)**		**U110**		**S(U53)**		**J53**
	E coli		***E coli***		***K pneumo***		***P aerug***		***K pneumo***		***E coli***		***K pneumo***		***P aerug***		***E coli***		***E coli***
MIC (μg/ml)	D	T	D	T	D	T	D	T	D	T	D	T	D	T	D	T	D	T	R
IPM	8	0.25	> 64	0.12	16	0.12	4	0.12	4	0.25	8	0.25	4	0.25	4	0.25	4	0.25	0.12
MPM	8	4	> 64	4	4	4	4	4	4	2	> 64	4	4	4	8	8	> 64	4	< 0.03
ETP	16	4	> 64	4	16	4	NA	NA	8	8	> 64	4	16	4	NA	NA	> 64	4	< 0.03
CAZ	> 64	32	> 64	32	> 64	32	> 64	32	> 64	32	> 64	32	> 64	32	> 64	32	> 64	32	< 0.03
CIP	32	8	> 64	8	> 64	8	> 64	8	> 64	8	> 64	8	> 64	8	> 64	8	> 64	8	< 0.03
AZT	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	0.03
NAL	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	> 64	1
COL	> 64	32	> 64	32	> 64	32	> 64	32	> 64	32	> 64	32	> 64	32	> 64	32	> 64	32	0.12
AK	32	2	> 64	2	64	2	64	2	64	2	64	2	64	2	64	2	64	2	1
CHL	> 64	4	> 64	4	> 64	4	> 64	4	> 64	4	> 64	4	> 64	4	> 64	4	> 64	4	2
GEN	> 64	32	> 64	32	> 64	32	> 64	32	> 64	32	> 64	32	> 64	32	> 64	32	> 64	32	0.5
Phenotype																			
ESBL	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	NA
Carba	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NA
Transferred genes																			
OXA-1	+	+	-	-	-	-	+	+	-	-	+	+	-	-	-	-	-	-	NA
SHV-1	+	+	-	-	-	-	+	+	-	-	+	+	-	-	-	-	-	-	NA
CTX-M-15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	NA
aac-lb-6'-cr	+	+	+	-	+	+	+	+	-	-	-	-	-	-	+	-	+	+	NA

IPM – imipenem; MPM – meropenem; ETP – ertapenem; CAZ – ceftazidime; CIP – ciprofloxacin; AZT – azithromycin; NAL – nalidixic acid; COL – colistin; AK – amikacin; CHL – chloramphenicol; GEN – gentamicin; ESBL – extended-spectrum beta-lactamases; Carba – carbapenemase (based on CarbaMP test); NA – not applicable; U – isolates from University College Hospital; I – isolates from Obafemi Awolowo Teaching Hospital; S – isolates from carrier isolates; D – donor; T – transconjugant; R – recipient; MIC – minimum inhibitory concentration; J53 – a derivative of *E coli* K-12 which is resistant to sodium azide widely used as a general recipient strain for various conjugation experiments.

Transfer of β-lactam resistance using ampicillin for selection was attempted from 13 of the 27 ESBL-carrying, carbapenem-resistant isolates, including susceptibility (MIC) profiles and clonality. Successful transfer was achieved from nine of these 13 isolates. The transconjugants recovered carried various genes including narrow-spectrum beta-lactamases, ESBLs and PMQRs; importantly, they all carried the *bla*CTX-M-15 gene (Table 1). The transconjugants demonstrated varying MICs of carbapenems; meropenem and ertapenem MICs ranged from 0.03 µg/ml to 8 µg/ml. Varying resistances to other antimicrobial agents were also transferred with the plasmids, including resistance to aztreonam (≥ 64 µg/ml), gentamicin (32 µg/ml), ciprofloxacin (8 µg/ml) and ceftazidime (32 µg/ml). However, it is noteworthy that high-level colistin resistance was also transferred through the entire isolates with MIC 32 µg/ml (Table 1). None of the plasmids were typeable for replicon/incompatibility group.

The steady-state concentration of drug taken up by the isolates exhibiting ESBL showed overall that the isolates did not accumulate less Hoechst 33342 than control isolates. Also, there was little variation in accumulation between the isolates, regardless of MIC profile, with all isolates accumulating relatively low levels of Hoechst 33342. *Escherichia coli* isolates were associated with either reduced expression or complete loss of OmpC. There was no loss of OmpF in any of the isolates. Similarly, *K pneumoniae* isolates were found to have complete loss of both OmpK35 and OmpK36 (Table 2).

DISCUSSION

There had been numerous global reports of carbapenem resistance in GNB including in some African countries (20, 21), but only recently has any information emanated from Nigeria (8). The focus of this study was to determine the mechanism of resistance to carbapenems from non-carbapenemase producing isolates. This study showed a high level of resistance to carbapenems associated with resistance to other classes of antimicrobial drugs in unrelated isolates of Gram-negative pathogens. However, carbapenem-resistant isolates were ESBL producers with no carbapenemase or AmpC enzymes detected. Therefore, carriage of an ESBL (in conjunction with loss of OMP) was a major contributor to phenotypic carbapenem resistance in this sample of isolates. These isolates all carried *bla*CTX-M-15. Other studies have incriminated an association of decreased outer membrane permeability with over-expression of β-lactamases possessing very weak carbapenemase activity, with the combination being sufficient to express phenotypic carbapenemase resistance (22, 23).

Various genes and resistance phenotypes were transferred from the carbapenem-resistant strains by conjugation. The transconjugants had MICs of carbapenem ranging from 0.03 µg/ml to 8 µg/ml. Meropenem and ertapenem showed very weak activity against the transconjugants, while imipenem and amikacin retained good activity against all the transconjugants. The variation in the MICs seen may reflect the additional carriage of other beta-lactamases in the original donor strains that were then detected in the transconjugants, or possible carriage of currently unrecognized genes that can also contribute to the phenotype. Varying high-level resistances to other classes of antimicrobial agents were also transferred with the plasmids, including aztreonam, colistin, ciprofloxacin and ceftazidime. Colistin/polymixin resistance had hitherto been considered

Table 2: Outer membrane protein profile of *Escherichia coli* (*E coli*) and *Klebsiella pneumoniae* (*K pneumoniae*) species

Identifier	Isolate	Outer membrane protein			
		F	C	A	K
U58	*E coli*	Present	RP	Present	Not applicable
S21	*E coli*	Present	Absent	Present	Not applicable
S(U25)	*E coli*	Present	RP	Present	Not applicable
S(U53)	*E coli*	Present	RP	Present	Not applicable
I364	Wild type *E coli*	Present	Present	Present	Not applicable
I88	*K pneumoniae*	Not applicable	Not applicable	Not applicable	Absent
U61	*K pneumoniae*	Not applicable	Not applicable	Not applicable	Absent
S(U37)	*K pneumoniae*	Not applicable	Not applicable	Not applicable	Absent
H43	Wild type *K pneumoniae*	Not applicable	Not applicable	Not applicable	Present

Outer membrane proteins F, C and A – outer membrane proteins in *E coli*
Outer membrane protein K – outer membrane proteins K35 and OmpK36 are outer membrane proteins in *K pneumoniae*
RP – reduced presence compared to wild type

chromosomal until recently when transferable plasmid-mediated resistance was inadvertently discovered in this study. This has also been reported during a routine surveillance project on antimicrobial resistance in commensal *E coli* from food animals in China (24).

It is important to note that various mechanisms of resistance to carbapenems exist in GNB in Nigeria. In addition to the mechanism described in this study, our previous study had also described the presence of known carbapenemases: NDM, VIM or GES (8). This study shows carbapenem resistance mediated by carriage of *bla*CTX-M-15 in tandem with either complete loss or reduced OMPs in a significant number of isolates without carbapenemase carriage. High-level colistin resistance was inadvertently observed in the entire isolates, and this calls for further studies on plasmid-mediated colistin resistance.

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